RECOMBINANT ADENOVIRUS

Field of the invention

The present invention relates to new recombinant adenovirus with changed tropism. More particularly the recombinant adenovirus has been constructed by removing the native knob structure and replacing it with a new cell binding ligand and an external trimerisation motif. The invention also relates to the new adenovirus for treatment of human diseases. Also included is a method for rescuing of recombinant adenovirus fibers into the adenovirus genome.

Background of the invention.

Clinical gene therapy was introduced in 1989. The aim at that time was to correct a gene defect in the immune system through the in vitro introduction of a healthy gene into the defect cells of the patient and transfusion of the treated cells back to the patient. Since that time, the possible indications for gene therapy have increased dramatically. Today, ten years after its introduction, the use of gene therapy to treat e.g. diseases of the blood vessels, cancer, inflammatory diseases and infectious diseases such as HIV can be envisaged.

At present, however, gene therapy is not a useful method in human medicine. One main reason is that gene therapy demands the packaging of the genes to be delivered into gene-carriers, or vectors, which can be injected into

patients and which will target the genes only to the intended cells. Such vectors have so far not been available.

Adenoviruses (Ad) are DNA viruses without an envelope, shaped as regular icosahedrons with a diameter of 60-85 nm. Cell-binding takes place through fiber proteins, anchored to the virion at the corners of the icosahedron. The fiber protein is not necessary for assembly and release of intact virions. Assembly of virions take place in the nucleus of infected cells.

The fiber protein, which is a homotrimer of a fiber polypeptide, contains three functionally different parts: an N-terminal tail anchoring the fiber non-covalently to the penton base in the virion and which furthermore contains the nuclear-localization signal; an approximate 15 amino acid fiber shaft motif which is repeated six times in Ad3 and 22 times in Ad2 and Ad5 (Chrobozek J, Ruigrok RWH and Cusack S: Adenovirus Fiber, *Current Topics in Microbiology and Immunology*, 1995, p 163-200); and a C-terminal globular domain, the knob, which contains the ligand which binds to the cellular Ad-receptor (See review in in the previous ref.). Each shaft repeat has two three-amino acid regions which form β-sheets and two amino acid regions which constitute the turns of the native extended fiber shaft. The crystal structure of the trimerised, cell-binding domain has been determined and shows a unique topology different from other anti-parallel β-sandwiches (Di Xia, Henry LJ, Gerard RD and Deisenhofer J: Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 Å resolution, *Structure* 2: 1259-1270,

1994.). Binding of the fiber to the penton base of the virion can take place also in a cell-free system, i.e. the fiber can bind to fiberless virions (Boudin M-L and Boulanger P: Assembly of Adenovirus Penton Base and Fiber, *Virology,* 116: 589-604, 1982).

It seems possible that the fiber can tolerate structural modifications as long as the ability to bind to the penton base and to be transported to the nucleus is retained. Some attempts at modifying the Ad fiber in order to change the binding properties of the virus have been made. A short peptide ligand has been added C-terminally of the knob (Michael SI, Hoy JS, Curie DT and Engles JT: Addition of a short peptide ligand to the adenvorirus fiber protein. *Gene Therapy 2: 660-8, 1995.*) and an octapeptide has been introduced into one of the knob "loops". By introducing the FLAG tetra-amino acid motif into the Ad penton, it has been shown possible to target Ad to cells normally not infected by Ad. This was done by targeting with bi-specific antibodies where one specificity was directed against the FLAG peptide and the other against the new target cell (Wickham TJ, Segal DM, Roelvink PW, Carrion ME, Lizonova A, Lee GM and Kovesdi I: Targeted Adenovirus Gene Transfer to Endothelial and Smooth Muscle Cells by Using Bispecific Antibodies. *J. Virol., 70: 6831-6838, 1996.*). It would therefore seem possible to target Ad to a broad range of human cells which would be very useful for the purpose of human gene therapy. For these reasons and for the reason that Ad have been used extensively for gene therapeutic applications (Trapnell BC and Gorziglia: Gene therapy using adenoviral vectors, *Current Opinion in Biotechnology,* 5: 617-625, 1994.) a method has now been

developed to create recombinant re-targeted Ad-virus which can be useful for human gene therapy.

Accordingly it is an object of the present invention to provide a recombinant adenovirus with changed tropism.

Another object of the invention is the recombinant adenovirus for treatment of human diseases.

A further object of the invention is a method for rescuing of recombinant adenovirus fibers into the adnovirus genome.

Summary of the invention

The objects of the invention are obtained by the recombinant adenovirus and the method for rescuing the virus fibers as claimed in the claims.

According to the invention there is provided a recombinant adenovirus with changed tropism. The adenovirus is characterized in that the native pentone fibre, which comprises a fibre tail, a fibre shaft and a fibre knob including a trimerisation motif, has been changed in that the native knob containing the cell binding structure and the native trimerisation motif has been removed and a new cellbinding ligand and an external trimerisation motif have been introduced into the virus fiber.

The structural modification has been performed by DNA technology at the gene level or by chemical or immunological means at the virus level.

According to another aspect of the invention adenovirus, as identified above, is used for the treatment of human diseases, either in vivo or by in vitro methods.

A further aspect of the invention is a method for rescuing of recombinant adenovirus fibers into the adenovirus genome comprising the following steps:

a) subcloning of a 9kb fragment (from Spe1 to end of genome),
b) further subcloning of a 3kb fragment between Sac1 and Kpn1,
c) deletion of the fibergene between Nde1 and Mun1 and replacing the missing sequence with SEQ ID NO: 13 in the Sequence listing containing an Xho1 site;
d) ligation of recombinant fiber between Nde1 and Xho1 of construct under c) above;
e) re-introduction of construct under d) above into the 9 kb fragment cut with Nhe1 using homologous recombination in E. coli;
f) isolation of the recombinant 9 kb fragment under e) and re-creation of the adenovirus genome by joining 9 kb fragment to the 27 kb fragment from the beginning of the genome to the Spe1 site by Cosmid cloning.

Detailed description of the invention

Figure legends

Fig. 1: Summary of modifications to native fiber carried out in the invention.

Fig. 2: Recombinant adenovirus fibers.

Fig. 3: Method for rescuing of recombinant fiber genes into the Ad genome.

Fig. 4a: Recombinant fibers rescued into Ad genomes which are capable of giving CPE/plaques on transfected cells and in secondary cultures.

Fig. 4b: Recombinant fibers rescued into Ad genomes which are capable of giving CPE/plaques on transfected cells and in secondary cultures.

In the present invention re-targeting of Ad is achieved through the introduction of a new cell-binding ligand into the fiber (Fig. 1). Any cell binding peptide can be used, e.g. a monoclonal antibody or a fragment thereof whether as a single chain fragment or Fab, a T cell receptor or a fragment thereof, an integrin binding peptide such as RGD or a growth factor such as Epidermal Growth Factor.

Ligands which so far have been applied include Epidermal Growth Factor (EGF), the amino acid motif RGD, a single chain fragment of a cloned T-cell receptor (scTCR) reactive with MAGE-1 peptides associated with HLA-A1 (vd Bruggen P, Traversaari C, Chomez P, Lurquin D, De Plaen E, vd Eynde B, Knuth A and Boon T: A Gene encoding an Antigen Recognized by Cytolytic T Lymphocytes on a Human Melanoma, *Science* 13 December 1991, 1643-1647.), a single chain fragment (scFv) of the monoclonal antibody G250, which with high selectivity has been shown to react with a protein antigen on human renal carcinoma cells (Oosterwijk E, Ruiter DJ, Hoedemaeker PhJ, et al: Monoclonal antibody G250 recognizes a determinant present in renal-cell carcinoma and absent from normal kidney. *Int J Cancer* 38: 489-94, 1986.). G250 has been extensively evaluated and has been applied in clinical trials (see the previous ref.).

Ad vectors can be made replication competent or incompetent for permissive cells. For tumor therapy, replication competent Ad has the potential advantage that it can replicate and spread within the tumor (Miller R and Curiel DT: Towards the use of replicative adenoviral

vectors for cancer gene therapy, *Gene Therapy* 3: 557-559). This may theoretically result in an increase of the chosen effector mechanism over that obtainable with replication incompetent vectors. Furthermore, infectious virus may contribute to an anti tumor effect by cytopathogenic effects in infected cells as well as by evoking an anti viral immune response which may harm infected cells.

Construction, expression and evaluation of recombinant fibers

The aim has been to develop a universal method for the construction of functional Ad fibers with changed binding-specificity to make possible the construction of re-targeted Ad.

The adenovirus fiber peptide carries several biological functions which are necessary to retain in order to produce active virus particles. The following fiber features are deemed to be of key importance in the construction of functional recombinant fiber peptides:

- The ability to form parallel homotrimers. This function is carried by the N-terminal amino acid sequence of the wild type fiber knob and is necessary for the fiber to be able to bind to penton base and to form the functional cell binding knob.
- The ability to bind to penton base to form penton capsomeres. This function is carried by the wild type fiber tail.
- The ability to express a cell-binding ligand allowing for attachment to target cells. This function is carried by the wild type fiber knob.

- Since adenovirus is assembled in the nucleus of infected cells, the ability to be transported into the nucleus of infected cells is vital to virus formation. The nuclear localization signal is mainly, but perhaps not exclusively, carried by the wild type fiber tail.

In the first stage recombinant fibers are constructed and evaluated in vitro after cell-free expression in a coupled transcription/translation system. Analysis by SDS-PAGE and autoradiography is performed to reveal the presence of an open reading frame and give information on the size of the translated product. In the next stage recombinant fibers are cloned into Baculovirus and expressed in insect cells allowing for functional studies of the fibers. Such studies include ability to form trimers as evaluated by immunostaining with monoclonal antibody 2A6.36 which has been shown to react only with trimerised fibers (Shin Hong J and Engler JA: The amino terminus of the adenovirus fiber protein encodes the nuclear localization signal, *Virology* 185: 758-767, 1991), expression of functional ligand as evidenced by ability to bind to cells expressing the corresponding receptor and ability to bind to penton-base either in solution or on virions.

Recombinant fibers are constructed using methodology based on PCR (Clackson T, Güssow D and Jones PT: General application of PCR to gene cloning and manipulation, in PCR, A Practical Approach, Eds McPherson MJ, Quirke P and Taylor GR, IRL Press, Oxford, p 187, 1992), e.g. PCR-ligation-PCR (Alvaro Ali S, Steinkasserer A: PCR-ligation-PCR Mutagenesis: A Protocol for Creating Gene Fusions and Mutations, *BioTechniques* 18: 746-750, 1995)

and splicing by overlap extension (SOE) (Horton RM and Pease LR: Recombination and mutagenesis of DNA sequences using PCR, in McPherson MJ (ed), Directed Mutagenesis, IRL Press 1991, p 217.). Cloning is performed according to standard methods. Recombinant fibers are sequenced using Perkin Elmer ABI Prism and are expressed in mammalian cells and in insect cells and stained with monoclonal antibodies specific for fiber tail, trimeric fiber and the new cell-binding ligand. The following parameters are evaluated after immunostaining:

- trimerisation
- nuclear transportation
- expression of the new cell-binding ligand.

Finally, recombinant fibers are rescued into the Ad genome by a newly invented procedure described below and recombinant virus particles are produced.

The invention will be further illustrated with the following non-limiting examples:

Example 1:

Fiber peptides are made where the knob is replaced with an external trimerisation motif which is introduced after the TLWT motif which ends the shaft portion of the fiber. The purpose behind the introduction of an external trimerisation motif is two-fold: a) to remove the knob containing the native trimerisation signal but also the cell-binding part of the fiber, and b) simultaneously to supply the necessary trimerisation signal. Two different amino acid motifs have been used, i.e. the 36 amino acid "Neck Region Peptide" = NRP (SEQ ID NO: 1 in Sequence

listing) from human "Lung Surfactant Protein D" (. Hoppe H-J, Barlow PN and Reid KBM: A parallel three stranded -helicalbundle at the nucleation site of collagen triple-helix formation. *FEBS Letters* 344: 191-195 (1994).) and a 31 aa "Zipper" motif where the leucine residues on positions 1 and 4 have been replaced with isoleucine residues = pII (SEQ ID NO: 2 in Sequence listing) (Harbury PB, Tao Zhang, Kim PS and Alber T: A Switch Between Two-, Three-, and Four-Stranded Coiled Coils in GCN4 Leucine Zipper Mutants. *Science* 262: 1401-1407, 1993.).

The DNA sequences for these trimerisation motifs are synthesized, cloned and sequenced in the project.

To replace the cellbinding function of the knob a new cellbinding ligand is introduced into the fiber in addition to the external trimerisation amino acid motif.

To augment the efficiency of nuclear transportation of recombinant fibers an external nuclear localisation sequence is added to the fiber in some cases.

In further embodiments the fiber in addition contains sequences which increase the survival of the fiber in the cytosol of infected cells, thereby enhancing transportation into the nucleus and virus assembly. Such sequences are e.g. sequences that are present in the wild type knob or in SEQ ID NO: 10 - 12.

The following types of fibers are constructed using the methods mentioned above (see Fig 2). The sequence of the

wild type fiber is shown in the sequence listing as SEQ ID NO 14.

Type A

where the trimerisation motif is fused to the fiber gene downstream of the fiber shaft after the TLWT motif which constitutes the four first amino acids of the fiber knob or downstream of the second turn (Turn b) of any shaft repeat, the remaining shaft repeats having been removed. The new cellbinding ligand is introduced downstream of the trimerisation signal with an amino acid linker motif being added between the trimerisation signal and the cellbinding ligand.

Type B

similar to type A but with a linker motif introduced immediately upstream of the trimerisation signal.

Type C

where the trimerisation motif is introduced after the first shaft repeat and in turn followed the shaft repeats 17 through 21. The new cellbinding ligand is introduced downstream of the trimerisation signal with an amino acid linker motif being added between the trimerisation signal and the cellbinding ligand.

Type D

where the cellbinding ligand is introduced between the restriction sites Nhe1 and Hpa1 in the fiber shaft, with an amino acid linker being added both upstream and downstream of the ligand.

Type D/Δ

This is a variant of Type D where the fiber shaft downstream of the cellbinding ligand in Type D was removed. Type D and (D/Δ) are constructed with the normal knob and with the knob being replaced with an external trimerisation signal as in Types A and B.

Type E

which are similar to Type A but with part of the knob being retained immediately upstream of the external trimerisation motif.

The following amino acid motifs are used as linkers in the above fiber constructs:

- SEQ ID NO: 3, derived from Psedomonas exotoxin
- SEQ ID NO: 4, derived from tissue prothrombin activator
- SEQ ID NO: 5, derived from the hinge region of mouse immunoglobulin
- SEQ ID NO: 6, derived from Staphylococcal protein A
- SEQ ID NO: 7, derived from the hinge region of human IgG3
- SEQ ID NO: 8, derived from shaft repeat no 17 of human Ad5

Recombinant fibers are cloned into Baculovirus and expressed in Sf9 cells and/or cloned into the vector pSecTag and expressed in COS cells as secreted proteins. Expression is monitored by immunostaining with monoclonal antibodies 4D2.5 (anti Ad5 fiber) and 2A6.36 (anti trimerised Ad5 fiber). Expression and trimerisation is

obvious in all recombinant fibers irrespective of length and trimerisation motif.

The various fibers which have been constructed and shown to be able to form trimers and express the new cell binding ligand are shown in Table 1. The results show that the invented technology is capable of generating trimerising fibers which express a new cellbinding ligand. It should therefore be possible to make functional virus with such fibers.

Table I. Results from immunostaining of different recombinant fibers

	Detecting antibody				
Fiber	**4D2**	**2A6**	**a-EGF**	**a-Ig**	**a-Id**
Type A					
A1 RGD	+	+			
A1 EGF	+	+	+		
A1 G250 HK	+	+		+	+
A1 G250 KH	+	+		+	+
A1 G250 KHJCH2	+	+		+	+
A1 VαLVβCβ	+	+			
A7 RGD	+	+			
A7 EGF	+	+	+		
A7 G250 HK	+	+		+	+
A7 G250 KH	+	+		+	+
A7 G250 KHJCH2	+	+		+	+
A7 VαLVβCβ	+	+			
A7 IgG3 EGF	+	+	+		
A7 (Gly4Ser)4 G250VKVH	+	+		+	+
A22 EGF	+	+	+		
A22 RGD	+	+			
Type B					
B (Gly4Ser)4 RGD	+	+			
Type C					
C IgG3 EGF	+	+	+		

	C (Gly4Ser)4-G250VKVH	+	+		+	+
	Type D					
	N/D EGF	+	+	+		
	N/D G250 HKCKγ	+	+		+	+
	F2/D EGF	+	+	+		
	F3/D EGF	+	+	+		
	Type D/Δ					
	F2 D/Δ G250 HKCK	+	+	+		
	F2 D/Δ G250 HKCKγ	+	+		+	+
	F2 D/Δ EGF	+	+	+		
	F3 D/Δ EGF	+	+	+		

Abbreviations used in Table 1:
2A6: antibody against trimerized fiber
4D2: antibody against fiber
a-EGF: antibody against epidermal growth factor
a-Id: anti idiotypic antibody specific for G250
a-Ig: antibody against mouse immunoglobulin
Cβ: Constant domain from β chain of T cell receptor against MAGE1/HLA A1. SEQ ID NO: 11.
CH2: immunoglobulin heavy chain constant domain 2
EGF: epidermal growth factor
G250: monoclonal antibody specific for renal carcinoma
H: heavy chain variable sequence from G250 (SEQ ID NO: 15)
IgG3:amino acid linker derived from hinge region of human IgG3, SEQ ID NO: 7
J: immunoglobulin joining chain sequence
K: light chain variable sequence from monclonal antibody G250 (SEQ ID NO: 16)
RGD: The amino acid sequence arginine-glycine-aspartic acid
Vα: Variable domain from α chain of T cell receptor against MAGE1/HLA A1. SEQ ID NO: 10
Vβ: Variable domain from β chain of T cell receptor against MAGE1/HLA A1. SEQ ID NO: 12

Example 2:

Nuclear localization of recombinant fibers (Tables 2 and 3)

Nuclear localization is assessed by immunostaining of fibers in Sf9 cells 24 hours after infection with the relevant Baculovirus clone. Some results are shown in Table 2 below. It is clear from these experiments that some recombinant fibers show a grossly impaired nuclear localization in Sf9 cells despite the presence of the nuclear addressing signal in the fiber tail.

Table 2
Nuclear localization of native and selected recombinant fibers in Sf9 cells

Fiber	% of fiber-expressing Sf9 cells showing nuclear localization after infection
Wild type	100
N/D EGF	100
A RGD	App. 50
A7 RGD	App. 100
A7 EGF	App. 100
A7 scTCR	App. 50
A7 G250 scFvs	0

Recombinant and native fibers have also been expressed in COS cells, targeted for expression in the cytosol after cloning into the vector pcDNA 3.1. In this case it was expected that the fibers would be detected in the nucleus, due to the presence of the native nuclear localization signal in the fiber tail. However, nuclear localization has so far only been detected in the wild type fiber and in fibers with single-chain T-cell

receptors, i.e. the fibers which have produced the most efficient virus (se below).

Since nuclear localization of fibers are crucial to virus assembly, an attempt is made to improve the efficiency of nuclear addressing by adding an external nuclear localization signal (NLS), in this case the SV40 large T-antigen NLS having the amino acid sequence SEQ ID NO: 9 (Fisher-Fantuzzi L and Vesco C: Cell-Dependent Efficiency of Reiterated Nuclear Signals in a Mutant Simian Virus 40 Oncoprotein Targeted to the Nucleus. *Mol Cell Biol*, 8:5495-5503, 1988). The external NLS sequence is added immediately up-stream of the RGD motif. It is found that the presence of the external NLS dramatically improved the nuclear localization in the cases where it has been investigated. In fact, as mentioned above the fiber constructs lacking the external NLS were undetectable in the transfected cells (Table 3).

Table 3
Nuclear localization of native and selected recombinant fibers in COS cells after targeting for expression in the cytosol

Fiber	Nuclear localization
Wild type	+
A VαLVβCβ	+
A VαLVβCβCk	+
A RGD	-
A NLS RGD	+
A7 RGD	-
A7 NLS RGD	+
A22 RGD	-

For abbreviations, se Table 1

The evidence given above support the hypothesis that recombinant fibers are poorly transported into the nucleus despite the presence of the intact tail region (see also below) and that this may possibly be corrected by the incorporation of an external NLS in the fiber construct.

Example 3:

METHOD FOR RESCUING OF RECOMBINANT FIBERS INTO VIRIONS

The wild type fiber in the Ad genome is exchanged for recombinant fibers by the following method (see Fig 3).

The plasmid pTG3602 (Chartier C, Degryse E, Gantzer M, Dieterlé A, Pavirani A and Mehtali M: Efficient generation of Recombinant Adenovirus Vectors by Homologous Recombination i Escherichia Coli, *J Virol*, 70: 4805-4810, 1996) containing the entire Ad5 genome as a Pac1-Pac1 fragment is used as starting material. The approximate 9kb fragment of the genome between Spe1 and Pac1 and containing the wild type fiber gene is cloned separately in pBluescript. From this fragment an approximate 3kb fragment between Sac1 and Kpn1 is further subcloned. A deletion of the native fiber gene with the exception of the N-terminal nucleotides upstream of the Nde1 site of the fiber, between the Nde1 site and the Mun1 site, which begins at base 38 after the stop codon of the fiber, is created in the 3kb fragment. The deleted sequence is replaced with SEQ ID NO: 13 which restores the Nde1 and Mun1 sites and the wild type genome sequence between the fiber stop codon and the Mun1 site. In addition the added sequence, SEQ ID NO: 13, contains an Xho1 site allowing for ligation of recombinant fibers

into the fiber-deleted 3kb fragment (the 3 kb fiber shuttle) between Nde1 and Xho1.

The 3 kb fiber shuttle with recombinant fiber is re-introduced into the 9 kb fragment cut with Nhe1 using homologous recombination in E.coli (see ref. in previous passage). The resulting recombinant 9 kb fragment is finally excised from the vector with Spe1 and Pac1 and joined to the isolated 27 kb fragment by Cosmid cloning.

The presence of an insert of the expected properties is verified in all cosmid clones by PCR. Cosmid clones are also restricted with Hind III and the presence of restriction fragments of the expected size verified on gels.

Recombinant Ad genomes are isolated after restriction with Pac 1 and used to transfect suitable cells. The occurrence of plaques is determined by microscopic inspection of the transfected cell cultures.

Supernatants are harvested from primarily transfected cultures and used to infect secondary cultures. The occurrence of cytopathogenic effects and plaques are monitored by microscopy.

The particular fiber constructs that have been successfully rescued into virus are shown in figure 4a and 4b.

Conclusion:

For gene therapy to be useful for treatment of human diseases there is a need for injectable vectors with ability to target specific cells or a specific tissue (Miller N and Vile R: Targeted vectors for gene therapy. *FASEB J,* 9: 190-199, 1995).

The present invention describes methods whereby knobless, trimerisation-competent fibers with new cellbinding ligands can been created and rescued into virus and have identified locations within the fiber-shaft which tolerates inserts of foreign ligands. The importance of intracellular trafficking of recombinant fibers has also been identified. Recombinant virus made using the invented technology should be highly useful in human medicine. Virtually unlimited opportunities for targeted gene-therapy may be developed by the combination of the technology described here and the identification of cell-binding ligands by phage-display.

So far trimerisation-competent fibers with a human scTCR have been and rescued into functional virus. Since single chain antibodies are large and highly complex peptides it seems highly likely that also other scAbs and cell-binding ligands, e.g. peptides identified from peptide libraries by means of phage-display, could be incorporated into Ad-fibers and rescued into virus using the same technology.

There are many ways in which Ad, made re-targeted by the present invention, may be applied to human gene therapy.

In the case of tumor diseases, the following options exist:

I. Use of vectors to introduce transgenes into tumors, such as

- anti onco genes
- "suicide" genes
- genes for immune modulatory substances or tumor antigens
- genes for anti angiogenetic factors

II. Use of infectious virus. This has the added value over the use of non replicating vectors that virus can spread from cell to cell within a tumor, thereby multiplying the initial hit on the tumor. Tumor cell destruction may occur not only by the cell-destroying mechanism engineered into the vector but also by the cell destruction which is associated with the virus infection per se and by the attack of the body's immune response on the virus infected cells. This principle has already been tested in man through the direct intra-tumoral injection of an adenovirus which has been made gene manipulated to replicate only in p53 mutant tumor cells. The experience from these limited trials on large "head-and-neck" tumors are partially encouraging with a complete regress of 2/11 treated tumors which are otherwise resistant to any form of known treatment.